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ORIGINAL ARTICLE

Differential Effects of Stress on Adult Hippocampal Cell Proliferation in Low and High Aggressive Mice

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Male wild house mice selected for a long (LAL) or a short (SAL) latency to attack a male intruder generally show opposing behavioural coping responses to environmental challenges. LAL mice, unlike SAL mice, adapt to novel challenges with a highly reactive hypothalamic-pituitary-adrenal axis and show an enhanced expression of markers for hippocampal plasticity. The present study aimed to test the hypothesis that these features of the more reactive LAL mice are reflected in parameters of hippocampal cell proliferation. The data show that basal cell proliferation in the subgranular zone (SGZ) of the dentate gyrus, assessed by the endogenous proliferation marker Ki-67, is lower in LAL than in SAL mice. Furthermore, application of bromodeoxyuridine (BrdU) over 3 days showed an almost two-fold lower cell proliferation rate in the SGZ in LAL versus SAL mice. Exposure to forced swimming resulted, 24 h later, in a significant reduction in BrdU + cell numbers in LAL mice, whereas cell proliferation was unaffected by this stressor in SAL mice. Plasma corticosterone and dentate gyrus glucocorticoid receptor levels were higher in LAL than in SAL mice. However, no differences between the SAL and LAL lines were found for hippocampal NMDA receptor binding. In conclusion, the data suggest a relationship between coping responses and hippocampal cell proliferation, in which corticosterone may be one of the determinants of line differences in cell proliferation responses to environmental challenges.

Key words: BrdU, coping style, dentate gyrus, hippocampal plasticity, neurogenesis.

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Male wild house mice selected for a long (LAL) or a short (SAL) latency to attack a male intruder in their home cage generally show profound differences in their behavioural responses to environmental challenges. For example, LAL mice show more freezing/immobility when exposed to uncontrollable or unescapable stressors, such as the shock-probe/defensive burying test (1), the two-way shock avoidance test (2), and the forced swim test (3, 4). Furthermore, LAL mice adapt more quickly to a 12-h shift in light/dark cycle and show more exploration in response to a small change in an otherwise familiar maze, whereas SAL mice rather show routine formation in both social and nonsocial situations (5–7). Thus, the LAL mice display a 'reactive' coping style, whereas SAL mice show a 'proactive' coping style (8, 9).

The reactive coping style of LAL mice corresponds with enhanced hypothalamic-pituitary-adrenal (HPA) axis reactivity when the mice are subjected to the acute stress of forced swimming or during exposure to a chronic psychosocial stressor (3, 4, 10, 11). The hippocampal mineralocorticoid receptor (MR)/glucocorticoid receptor (GR) mRNA expression ratio is decreased after chronic stress in LAL mice (12). Furthermore, in LAL mice, the hippocampal intra- and infra-pyramidal mossy fibres terminal fields are larger (13), which may be related to spatial learning capacity (14). In addition, several cytoskeleton genes, calmodulin-related genes and genes encoding mitogen activated protein kinase (MAPK) cascade components are expressed at higher levels in the hippocampus of LAL compared with SAL mice (15). These

findings suggest line-differences in adrenocortical responsiveness and hippocampal structural plasticity.

The proliferation of precursor cells and neurogenesis are important determinants of hippocampal plasticity. Neurogenesis continues to occur in the adult hippocampal dentate gyrus. It has been proposed that the continuous proliferation of new cells enables the hippocampus to adapt more readily to novelty and other challenges (16). Indeed, various positive correlations between adult proliferation and hippocampal-dependent learning have been reported (17–19). Moreover, elevated circulating glucocorticoid concentrations and stress are amongst the most potent inhibitors of dentate gyrus proliferation in various species and paradigms (20–26).

The SAL and LAL mouse lines have been selected on the basis of distinct behavioural and neuroendocrine phenotypes that represent the extremes of the behavioural response repertoire (27). Such extremes are valuable to understand better the mechanisms underlying interindividual differences in specific traits or phenotypes as they exist in a given population. The present study aimed to test the hypothesis that their distinct phenotypes are reflected in differences in hippocampal cell proliferation rate under basal and/or stressful conditions. For this purpose, basal cell proliferation rate was determined using immunocytochemistry for Ki-67, an endogenous DNA binding protein selectively expressed in cells engaged in cell cycle, except G₀ (28, 29). Furthermore, using the birth date marker 5-bromo-2'-deoxyuridine (BrdU) (30, 31) adult-generated cells were identified. Animals were killed 24 h after BrdU administration to allow at least one and possibly two cell cycles in the mouse. Hence, the term proliferation used in the present study may include the very early stages of maturation as well as proliferation *per se*. The measurements for neurogenesis were further related to circulating corticosterone concentrations, hippocampal GR protein levels and hippocampal NMDA receptor binding in both mouse lines.

Materials and methods

Mice

Male LAL and SAL mice originated from a colony of wild house mice (*Mus musculus domesticus*) maintained at the University of Groningen, the Netherlands, since 1971. The LAL males used for the experiments came from the 38–40th generation of selection and the SAL males came from the 66–68th generation (this difference in generation between LAL and SAL mice is the result of unsuccessful breeding of LAL mice at the beginning of the selection). The mice were housed in plexiglass cages (17 × 11 × 13 cm) in a room under a 12 : 12 h light/dark cycle (lights on 00.30 h). Standard laboratory chow and water was available *ad libitum*. Mice were weaned at 3–4 weeks of age, and were paired male–female at the age of 6–8 weeks and were housed in this way during all experiments.

At 14 weeks of age, male mice were subjected to the attack latency test (32). Briefly, a male was confronted with a standard non-aggressive male opponent in a neutral cage on three consecutive days. The attack latency score was taken as the mean of these daily scores. Neither LAL nor SAL mice experienced a social defeat. Non-attacking LAL mice and SAL mice with an attack latency of less than 50 s were used for the experiments. Male LAL and SAL mice used for this study were aged 18 ± 2 weeks. All experiments were in accordance with the regulations of the Committee for Use of Experimental Animals of the University of Groningen (DEC no. 2326).

Basal hippocampal cell proliferation

Ki-67

LAL and SAL mice (*n* = 8 per line) were left undisturbed in their home cage for at least one week. Thereafter, mice were rapidly perfused in the morning and numbers of proliferating cells were determined using Ki-67 immunocytochemistry as described below.

BrdU

LAL and SAL mice (*n* = 9 per line) received three i.p. injections of 10 mg/ml BrdU (daily dose: 50 mg/kg body weight, dissolved in sterile 0.9% NaCl, Sigma, St Louis, MO, USA), once per day for three consecutive days. Mice were perfused 24 h after the last injection. It should be noted that by measuring BrdU-positive cells 24 h after the last BrdU injection, BrdU-positive cells reflect not only proliferating cells but also cells undergoing very early stages of maturation. Blood was obtained from the heart just prior to perfusion to determine corticosterone concentrations.

Acute stress-effects on hippocampal cell proliferation

LAL (*n* = 10) and SAL (*n* = 8) mice received a single i.p. injection of 20 mg/ml BrdU (dose: 100 mg/kg body weight, dissolved in sterile 0.9% NaCl). Immediately afterwards, the mice were forced to swim in a narrow plexiglass cylinder (30 cm high, diameter of 10 cm) filled with water of 25 °C for 5 min. The depth of the water was 8.5 cm, which enabled the mice to reach the bottom with their tail. The forced swim test was chosen because this is a uniform and strong psychological and physical stressor, sufficient to generate stress-induced changes in cell proliferation. Importantly, because forced swimming was found to amplify behavioural and neuroendocrine phenotypes of SAL and LAL mice (3, 4, 33), this was taken into account as well. Control LAL and SAL mice (*n* = 8 per line) received the same dose of BrdU but were then left undisturbed. Twenty-four hours after the BrdU injection, blood was obtained from the heart prior to perfusion for plasma corticosterone measurement.

To determine the effect of the injection and/or swim stress exposure on the corticosterone response, an additional group of LAL and SAL mice received the same treatment as mentioned above, but were killed 15 min after exposure to forced swimming (*n* = 8 per line) or 15 min after the injection (control mice, *n* = 7 per line). Trunk blood was obtained by decapitation for plasma corticosterone determination.

Assessment of hippocampal GR protein levels and NMDA receptor binding

Hippocampal GR protein levels were measured by quantitative immunocytochemistry in the same brains of the naïve LAL and SAL mice (*n* = 8 per line) that were used for the Ki-67 immunostaining. An additional group of naïve LAL and SAL mice (*n* = 8) received an overdose of CO₂ anaesthesia, and brains were rapidly removed, quickly frozen in ice cold iso-pentane and stored in –80 °C for subsequent measurements of hippocampal NMDA receptor binding using autoradiography.

Tissue processing

For the cell proliferation experiments, mice received an overdose of CO₂ anaesthesia and were perfused transcardially with heparinised 0.9% saline followed by 4% paraformaldehyde in cold phosphate buffer (pH 7.4). The brains were postfixed overnight, transferred into 30% sucrose and stored

at 4 °C. After 24–48 h, coronal sections of 40- μ m thickness were cut on a freezing microtome. The sections were stored at –20 °C in cryoprotectant containing 25% ethylene glycol, 25% glycerin and 0.5 M phosphate buffer.

Radioimmunoassay for corticosterone

Blood samples were collected in chilled tubes containing EDTA, centrifuged at 2600 g for 10 min at 4 °C, and plasma samples were stored at –20 °C until assayed. Plasma corticosterone was determined in duplo using a commercially available radioimmunoassay kit (Mouse Corticosterone RIA Kit, ICN Biomedicals, Costa Mesa, CA, USA). The detection limit of the assay was 3 ng corticosterone/ml with an intra-assay variance of 4.4% and interassay variance of 6.5%.

Immunocytochemistry

Ki-67 immunocytochemistry

Ki-67 immunocytochemistry was performed as described earlier (25). Briefly, mounted sections were rinsed with 0.1 M Tris buffered saline pH 7.6 (TBS). Sections were placed in 2 plastic jars filled with citrate buffer (0.01 M, pH 6.0) and pretreated in a domestic microwave oven (800 W). Microwave treatment took 15 min in total, starting at 800 W for 5 min until boiling was reached, after which the setting was lowered to 260 W. Following 30 min of cooling at room temperature, non-specific binding was blocked by incubating in TBS + 2% milk powder (Campina Melkunie, Eindhoven, the Netherlands) for 30 min. Sections were then incubated overnight at 4 °C with polyclonal anti-Ki-67 (Novocastra, Newcastle, UK, 1 : 2000) in Supermix (TBS/0.25% gelatin/0.5% Triton X-100). Following rinsing in TBS, sections were incubated with biotinylated sheep anti-rabbit (Amersham Life Sciences, Den Bosch, the Netherlands, 1 : 200) in Supermix for 1.5 h, after which signal was amplified with ABC Elite (Vector Laboratories, Burlingame, CA, USA, 1 : 800) in TBS/BSA 1% for 2 h and again with biotinylated tyramide (1 : 500, kindly provided by Dr I. Huitinga, Netherlands Institute for Brain research, Amsterdam) and 0.01% H₂O₂ for 30 min followed by another 1.5 h incubation with ABC (1 : 1000). Colour development was performed with diaminobenzidine (0.50 mg/ml DAB, Sigma, 0.01% H₂O₂) for 10 min.

BrdU immunocytochemistry

BrdU immunocytochemistry was performed as described earlier (25, 34). Briefly, free-floating sections were treated with 0.6% H₂O₂ in TBS (pH 7.6) for 30 min, following incubation in 50% formamide/2 × SSC (0.3 M NaCl and 0.03 M sodium citrate) for 2 h at 65 °C, acidification/denaturation with 2 M HCl for 30 min at 37 °C and incubation in 0.1 M boric acid pH 8.5 for 10 min at room temperature. Sections were then incubated overnight at 4 °C with mouse anti-BrdU (Novocastra; 1 : 200) in TBS-plus (TBS/0.1% Triton X-100/3% normal horse serum). After washing with TBS-plus for 30 min, sections were incubated with biotinylated horse antimouse (1 : 200) for 45 min, followed by the streptavidin-HRP and DAB (0.25 mg/ml DAB, 0.01% H₂O₂).

GR immunocytochemistry

A well characterised GR-specific antibody was used (raised by M. Kawata, Kyoto, Japan) according to protocols described earlier (35). In brief, free floating sections were washed in 0.1 M PBS, 0.3% triton X-100, and incubated with GR antiserum (1 : 5000) for 48 h at 4 °C, washed and incubated with biotinylated anti-rabbit (1 : 200, Vector Laboratories) for 2 h, washed

again and then amplified with avidin-biotin peroxidase complex (ABC 1 : 1000, Vector Laboratories) for 1 h at room temperature. Chromogen development was performed with DAB (0.25 mg/ml, 0.01% H₂O₂) after which they were washed in PBS, mounted, dried and coverslipped with Entellan (Merck, Darmstadt, Germany). For quantification purposes, all incubations including the one in DAB were performed in a standardised manner, assuring all individual slides to receive exactly the same incubation time.

NMDA receptor autoradiography

Brain tissue sections of 20 μ m thickness were cut on a cryostat and thaw-mounted on gelatine-coated slides. These slides were stored at –80 °C until the time of radioligand binding. The sections were de-moisturised overnight in the presence of silica gel and stored at –80 °C. The labelling was performed at ligand concentrations saturating all binding sites. NMDA receptor radiography was described elsewhere (36). Sections were preincubated in a 50 mM Tris buffer (pH 8.0) for 3 × 15 min at room temperature. The incubation solution contained 50 nM [³H]CGP39653 (40.0 μ Ci/nmol, Amersham, NET-780), 2.5% GDH (L-glutamic dehydrogenase, Sigma), 1.135 mM NAD (β -nicotinamide adenine dinucleotide, Sigma) and 0.05% hydrazine (Sigma). The slices were incubated for 1 h at 4 °C. Subsequently, the slides were rinsed for 3 × 30 s in ice-cold preincubation buffer and for 3 s in distilled water. Finally, the preparations were dried and exposed to a ³H-sensitive film for 6 weeks. The films were developed with a Kodak D19 developer (5.8 mM Elon, 0.317 M Na₂SO₃, 0.082 M C₆H₆O₂, 0.358 M Na₂CO₃, 3.33 mM citric acid and 6.75 mM K₂S₂O₈), and were fixed with a 30% solution of Na₂S₂O₃·H₂O.

Stereological quantification of proliferation

Ki-67 and BrdU-labelled cells were counted in a stereological approach throughout the rostro-to-caudal extent of the entire hippocampus using a light microscope (× 400) by a researcher blinded to the study code. Ki-67+ and BrdU+ cells were scored in the SGZ (defined as a 2–3 cell-body-wide zone along the border of the granule cell layer with the hilus) as described previously (16, 25) in serially sampled, coronal 40- μ m sections. We intentionally avoided the Stereo Investigator method as it, for the quantification of 'rare' events, generates data with high variance (37). We quantified all BrdU+ and Ki-67+ cells in a modified stereological approach in randomised and coded 40- μ m sections from a systematically sampled 1-in-10 series through the entire rostro-to-caudal extent of the dentate gyrus. Ki-67+ cells were scored bilaterally, BrdU+ cells were scored unilaterally (basal cell proliferation) or bilaterally (stress-induced cell proliferation). Data shown reflect total cell counts per animal. In addition, the surface area of the granule cell layer was estimated in the same sections in which BrdU cell number had been assessed using an automated image analysis system (Quantimet 500, Leica, Cambridge, UK). The surface areas of the brain sections were summed per mouse and expressed as mm².

Quantification of GR-immunoreactivity

GR immunocytochemical signal was quantified using optical density (i.e. grey values) measurements of the pyramidal and granular cell layers, following previous descriptions by others (38). Briefly, three to four levels of the dorsal hippocampus per mouse were immunostained for GR and then digitised using a CCD camera connected to a microscope at × 2.5 magnification. Digitised images were stored on a Macintosh computer and analysed using 'Object-Image' software (<http://simon.bio.uva.nl/object-image.html>), which is an adaptation of NIH Image (<http://rsb.info.nih.gov/ni-image>). Following a standard normalisation procedure, mean optical densities of the GR

immunoreactive signal were determined in manually drawn outlines of the CA1-2 pyramidal and dentate gyrus granular cell layers. A standardised rectangle of $500 \times 700 \mu\text{m}$ was placed over the stratum radiatum, and the grey value determined in there was used for background subtraction. Corrected mean values were expressed per subregion and per animal and as ratio of CA1-2 over the dentate gyrus.

Quantification of NMDA receptor autoradiography

Densitometric analysis of receptor binding was performed with the use of a computer assisted image analysis system (Quantimet 600, Leica). The optical density of receptor labelling was measured in the stratum oriens and the stratum radiatum of the CA1 region, and in the inner and outer molecular layers of the dentate gyrus. For each animal, three consecutive coronal sections were measured and averaged. The optical density of receptor labelling was expressed as tissue equivalent (nCi/mg brain tissue) according to autoradiographic [^3H]-microscales (Amersham, RPA-506).

Statistical analysis

An unpaired Student's *t*-test was used to determine line differences in Ki-67+ cells and BrdU+ cells, surface area, corticosterone concentrations, GR-immunoreactivity and NMDA receptor labelling. Univariate analysis of variance (ANOVA) was used to determine line and treatment effects of forced swimming on BrdU+ cells and corticosterone concentrations. When significance was revealed, ANOVA was followed by a Bonferroni post-hoc test. For all tests the software package SPSS, version 11 (SPSS Inc., Chicago, IL, USA) was used. Data are presented as mean \pm SEM. $P < 0.05$ was considered statistically significant.

Results

Basal hippocampal cell proliferation

Ki-67

Ki-67+ cells were identified in the SGZ, dentate gyrus hilus, and in the CA1 stratum radiatum (Fig. 1A), often in clusters or doublets (Fig. 1B,C). Quantification of the number of Ki-67+ cells revealed a significantly lower number of cells in the SGZ in LAL compared with SAL mice ($P < 0.05$, Fig. 1D). Quantification of other hippocampal regions revealed that LAL mice had significantly more Ki-67+ cells in the CA1 stratum radiatum than SAL mice ($P < 0.05$; Table 1).

BrdU

LAL mice showed an almost two-fold lower total number of BrdU+ cells in the SGZ compared with SAL mice ($P < 0.001$; Fig. 2A). No line-difference was found for surface area of the granule cell layer (LAL: $0.98 \pm 0.04 \text{ mm}^2$; SAL: $1.01 \pm 0.02 \text{ mm}^2$). Importantly, 24 h after the last BrdU injection, plasma corticosterone concentrations were significantly higher in LAL mice than in SAL mice ($P < 0.05$; Fig. 2B). A typical example of BrdU labelling in the hippocampal dentate gyrus of a LAL and SAL mouse is depicted in Fig. 3.

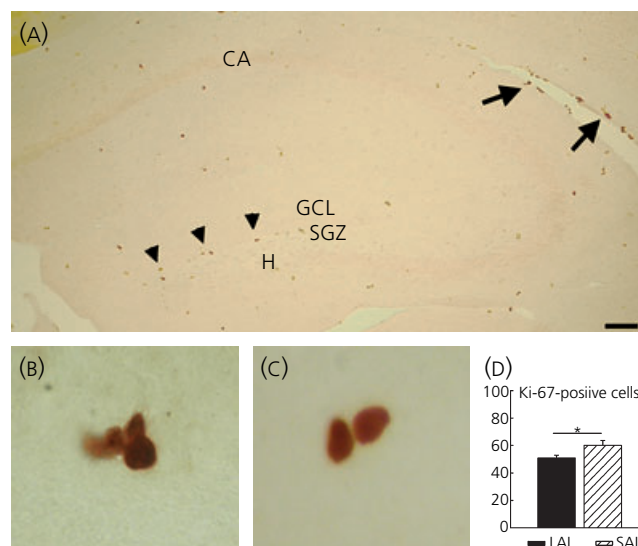


Fig. 1. Ki-67 labelling in tissue sections of the hippocampus revealed clearly positive cells mainly in the dentate gyrus hilus (H) and subgranular zone (arrowheads) subregions (A). Also in the subventricular wall, proliferating cells are abundantly present (arrows). Positive cells were also found in CA1 stratum radiatum layers (CA). Details of Ki-67+ clusters (B) or doublets (C) clearly represent dividing cells. The number of Ki-67+ cells in the SGZ was significantly lower in LAL compared with SAL mice (D). The numbers indicate total cell counts in both hemispheres \pm SEM. * $P < 0.05$, Student's *t*-test. GCL, granular cell layer. Bar represents $160 \mu\text{m}$.

Table 1. Ki-67+ cell numbers in hippocampal subregions in naïve LAL and SAL Mice.

	Mol cell layer	Dentate gyrus	CA1 rad	Hippocampus
LAL (n = 8)	27.5 ± 3.3	88.5 ± 2.9	$49.5 \pm 4.7^*$	138.4 ± 6.0
SAL (n = 8)	24.6 ± 2.4	95.4 ± 5.8	36.6 ± 3.3	133.1 ± 6.8

Ki-67+ cells were assessed in a stereological approach in serially sampled, coronal $40\text{-}\mu\text{m}$ sections in a 1-in-10 series throughout the entire rostro-to-caudal extent of the hippocampus. Subregions counted included the molecular cell layer of the dentate gyrus (mol cell layer), dentate gyrus (including subgranular zone, granule cell layer, hilus and molecular cell layer), CA1 stratum radiatum (CA1 rad) and per total hippocampus (including dentate gyrus, pyramidal cell layer of CA1-2, CA1 rad). The numbers indicate total cell counts in both hemispheres \pm SEM. * $P < 0.05$ versus SAL, Student's *t*-test.

Acute stress-effects on hippocampal cell proliferation

LAL mice showed more immobility behaviour during forced swimming than SAL mice (duration in percentage time: LAL, 71.5 ± 4.33 ; SAL, 35.5 ± 4.86 ; $P < 0.001$, Student's *t*-test).

Regarding cell proliferation, a line effect [$F(1,30) = 7.834$, $P < 0.01$] and a line \times treatment effect [$F(1,30) = 5.130$, $P < 0.05$] were found for the number of BrdU+ cells in the SGZ of the dentate gyrus. LAL mice subjected to forced swim stress showed a

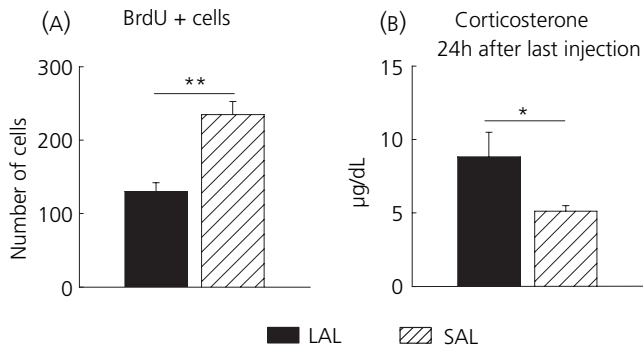


Fig. 2. Number of BrdU+ cells in the subgranular zone of the dentate gyrus (A) and plasma corticosterone concentrations (B) of LAL and SAL mice, 24 h after the third i.p. injection of BrdU. The numbers indicate total cell counts in one hemisphere + SEM. * $P < 0.05$, ** $P < 0.001$, Student's *t*-test.

significant decrease in BrdU+ cell number compared with control LAL mice ($P < 0.05$) and compared with SAL mice ($P < 0.005$) (Fig. 4A). Interestingly, no line difference in cell proliferation rate was found after a single injection of BrdU (i.e. control LAL versus control SAL mice).

Plasma corticosterone concentrations

Plasma corticosterone concentrations measured 24 h after forced swimming revealed a significant treatment effect [$F(1,29) = 4.442$, $P < 0.05$]. Corticosterone concentrations were significantly higher in stressed LAL mice compared with control LAL mice ($P < 0.05$; Fig. 4B). In an additional group of LAL and SAL mice, a treatment effect was found for plasma corticosterone measured 15 min after forced swimming [$F(1,26) = 10.811$, $P < 0.005$]. Exposure to forced swimming along with a single i.p. injection resulted in higher plasma corticosterone concentrations compared with a single i.p. injection only (significant difference in LAL, $P < 0.05$; tendency in SAL, $P = 0.053$; Fig. 4C).

Hippocampal GR-immunoreactivity

As described previously (35, 39, 40), GR-immunopositive nuclei were found in the cortex, amygdala, hypothalamus as well as hippocampus, with high densities in the dentate gyrus and CA1-2 regions (Fig. 5). In the CA3-4 pyramidal layers, almost all cells were devoid of nuclear GR signal. Quantification of mean optical densities within the hippocampus (Table 2) revealed a significant difference between LAL and SAL mice in the dentate gyrus ($P = 0.045$), but

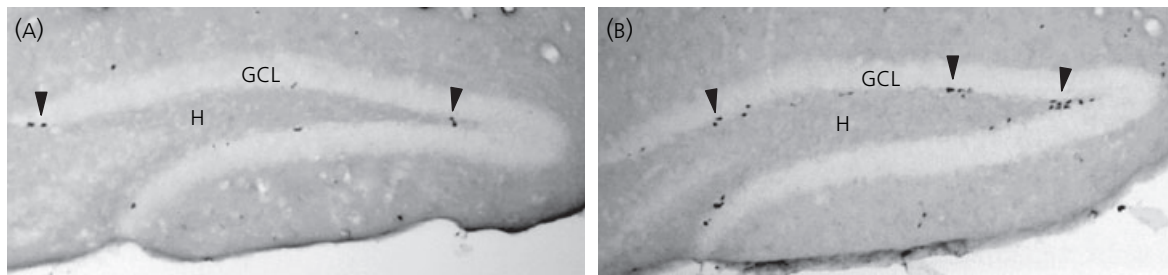


Fig. 3. BrdU labelling in tissue sections of the hippocampal dentate gyrus of a LAL mouse (A) and a SAL mouse (B), which were obtained 24 h after the third i.p. injection of BrdU. BrdU+ nuclei are clearly present in the subgranular zone (arrowheads). Note the lower number of BrdU+ cells in LAL compared with SAL mice. GCL, Granule cell layer of the dentate gyrus; H, hilus region of the dentate gyrus.

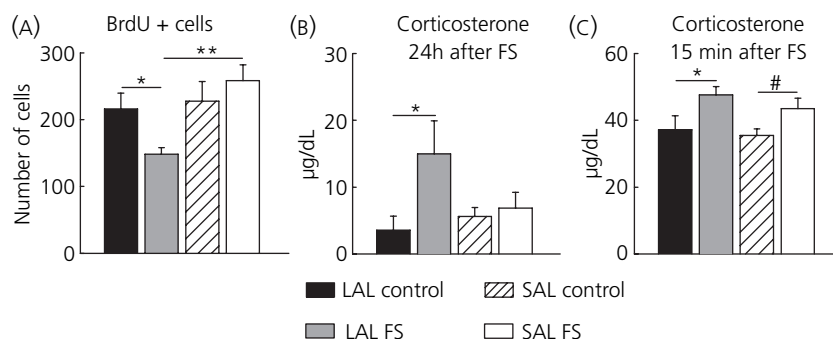


Fig. 4. Effects of 5 min of forced swimming of LAL and SAL mice on the number of BrdU+ cells in the subgranular zone of the dentate gyrus (A), and on plasma corticosterone concentrations 24 h later (B). Effects of 5 min of forced swimming following a single i.p. injection on plasma corticosterone concentrations in LAL and SAL mice 15 min later (C). The numbers indicate total cell counts in both hemispheres + SEM. * $P < 0.05$; ** $P < 0.005$, # $P = 0.053$, ANOVA followed by Bonferroni post-hoc test.

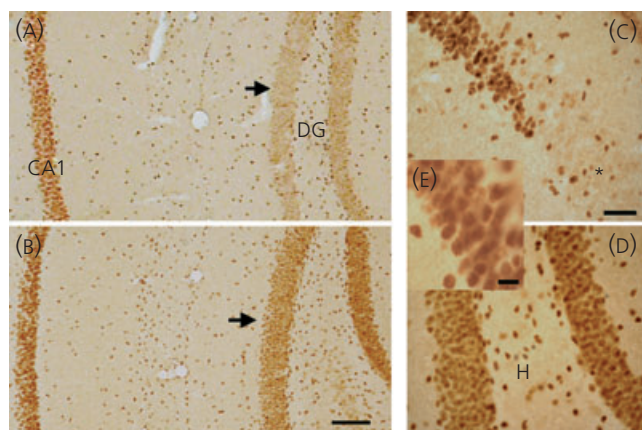


Fig. 5. Examples of glucocorticoid receptor (GR)-immunocytochemistry in the hippocampus of a SAL (A) and a LAL (B) mouse, showing prominent nuclear signal in the CA1 subregion and dentate gyrus. Higher GR-immunoreactivity is apparent in the dentate gyrus of the LAL mouse. (C) Showing a higher magnification of the nuclear GR immunoreactive signal in the CA1 subregion (upper left). The asterisk indicates the start of the CA3 subregion where the GR is known to be absent. (D) An example of the dentate gyrus at higher magnification; the inset (E) shows a detail of CA1 cells. (A,B) Scale bar = 120 μ m; (C,D) scale bar = 60 μ m; (E) scale bar = 18 μ m. DG, Dentate gyrus; H, hilar region.

not in the CA1-2 area ($P = 0.29$). Ratios of CA1-2 over dentate gyrus did not differ between both lines ($P = 0.17$).

Hippocampal NMDA receptor binding

No differences were found between LAL and SAL mice for NMDA receptor binding in the dentate gyrus or CA1 subregions (Table 3).

Discussion

The present study demonstrates that a genetic difference in behavioural adaptation is accompanied by altered hippocampal cell proliferation under both basal and acute stress conditions. Specifically, LAL mice showed less Ki-67+ cells and less BrdU+ cells in the SGZ of the dentate gyrus under baseline conditions than SAL mice. Furthermore, a significant reduction in the number of BrdU+ cells was observed 24 h after exposure to 5 min of forced swimming in LAL, but not in SAL mice. The higher plasma corticosterone levels and higher GR-immunoreactivity selectively in the dentate gyrus of LAL

Table 2. Optical Densities (Arbitrary Units) of Glucocorticoid Receptor-Immunocytochemistry in the Dentate Gyrus and CA1-2 Subregions of the Hippocampus of LAL and SAL Mice.

	Dentate gyrus	CA1-2 area
LAL (n = 8)	14.7 \pm 1.5*	25.1 \pm 1.0
SAL (n = 8)	10.8 \pm 1.2	23.2 \pm 1.6

Data are presented as mean \pm SEM. * $P < 0.05$ versus SAL, Student's *t*-test.

Table 3. Optical Densities (nCi/mg) of NMDA Receptor Binding in the Inner and Outer Molecular Layer of the Dentate Gyrus (DG) and in the CA1 Stratum Radiatum (CA1 rad) and CA1 Stratum Oriens (CA1 ori) of the Hippocampus of LAL and SAL Mice.

	DG inner	DG outer	CA1 rad	CA1 ori
LAL (n = 8)	16.9 \pm 1.0	16.6 \pm 1.2	23.9 \pm 1.0	22.0 \pm 1.1
SAL (n = 8)	19.3 \pm 1.3	18.8 \pm 1.0	24.8 \pm 1.3	22.3 \pm 0.8

Data are presented as mean \pm SEM.

mice may underlie, in part, this stress-induced decrease in hippocampal cell proliferation rate.

Basal hippocampal cell proliferation

Basal cell proliferation rate in the SGZ of the dentate gyrus, as studied with the endogenous marker Ki-67 (29), was found to be significantly lower in LAL compared with SAL mice. Using BrdU as an exogenous birth date marker for proliferation, significantly less proliferating cells were observed in the SGZ of LAL compared with SAL mice after three daily BrdU injections, but not after a single BrdU injection. Corticosterone levels were significantly higher 24 h after the third, but not after a single, BrdU injection in LAL mice. Therefore, the lower BrdU+ cell number in LAL mice likely reflects a line-difference in corticosterone response following the stress of repeated injections.

The different outcome between the BrdU+ cell number after a single BrdU injection and Ki-67+ cell number in LAL and SAL mice is puzzling. One factor that needs to be investigated in future studies is a possible line-difference in bio-availability of BrdU in brain due to clearance or blood brain barrier passage. Furthermore, the different outcome could be explained by methodological differences. BrdU cell number only reflects a snapshot following 2 h of BrdU bioavailability and identifies only cells in S-phase, whereas Ki-67 identifies cells present in all stages of the cell cycle except G₀. The final number of BrdU+ cells is influenced by the continuing division of the newborn cells and depends on the initial BrdU dose and the subsequent survival time after injection. Secondary generations of dividing cells will initially yield larger numbers of BrdU+ cells until the label becomes diluted below detection level. Moreover, a significant proportion of the newly generated cells die as well (41). As such, BrdU+ cells represent a very heterogeneous population at 24-h survival after a BrdU injection, that is difficult to directly compare to Ki-67 in absolute numbers, as discussed previously (29).

The present findings suggest that the higher expression of markers for hippocampal plasticity, as seen in the more 'reactive' LAL mice (13, 15), is linked with lower hippocampal cell proliferation at basal levels, as previously observed (42). However, hippocampal plasticity is strongly influenced by environmental factors. For example, animals reared in larger, more complex environments show enhanced dendritic branching and neurogenesis, and improved learning and memory (43–47). Conversely, environmental deprivation might cause a reduction in these structural and functional

features (47). The housing conditions of SAL and LAL mice are restricted (standard cage, standard rearing facilities, no environmental enrichments), which by itself might have affected the cell proliferation rate in the SGZ, especially in the LAL mice. Because LAL mice rather than SAL mice readily adapt to novel challenges (5, 6, 48), it is tempting to speculate that under conditions of environmental enrichment the cell proliferation rate might be higher in LAL compared with SAL mice. On the other hand, recent studies failed to demonstrate parallels between changes in (hippocampus-related) behaviour and alterations in adult hippocampal neurogenesis (49, 50). These findings might indicate that certain types of behavioural adaptation do not require hippocampal cell proliferation or neurogenesis. Further research is necessary to investigate whether this is true for the LAL mice as well.

It is important to note that by using a survival time of 24 h after BrdU labelling, it is not possible to distinguish between glia- and neurogenesis because no marker proteins for glia or neurones are coexpressed in such young cells. Similarly, Ki-67 is a marker that detects proliferating cells but not differentiating cells. Whether or not survival or selective phenotypes are affected, awaits future studies. Because no adult neurogenesis occurs in the CA1 stratum radiatum, it is likely that the higher number of Ki-67+ cells in LAL compared with SAL mice represent proliferating glia. Further studies are needed to investigate whether these glia cells might play a role in the differences in size of the mossy fibres terminal fields and in hippocampal gene expression profile between the LAL and SAL lines, as reported previously (13, 15).

Acute stress effects on hippocampal cell proliferation

Forced swim stress induced a selective decrease in hippocampal cell proliferation, as assessed 24 h later in the dentate gyrus of LAL mice. Stress-induced inhibition of cell proliferation has previously been described in several species including mice, rat, tree shrew and monkey (21–23, 25, 51, 52). Unlike chronic stress conditions that may cause dendritic atrophy and endanger hippocampal viability (53, 54), acute stress-induced morphological rearrangement of the hippocampus, like proliferation, is considered to be an adaptive response (55, 56). This is further emphasised by the observation that stress-induced reductions in cell proliferation are transient and reversible. Reduced cell proliferation upon stress can normalise again after a recovery period, following treatment with antidepressants, or following the application of a corticotrophin-releasing factor receptor antagonist, a vasopressin V1b receptor antagonist or a GR antagonist (25, 51, 57–60). Thus, the present stress-induced decrease in proliferation may indicate that, unlike SAL mice, LAL mice do respond to a stressor by means of structural adaptation. In addition to the previously established differences in coping style and HPA axis reactivity (3, 4, 6, 8, 9), this additional level of hippocampal plasticity appears unique for the LAL line and may be a possible prerequisite for their behavioural repertoire.

The swim stress-induced suppression of cell proliferation was associated with higher plasma corticosterone concentrations

15 min and 24 h poststress in LAL mice. Despite this, no individual correlation was found between 24-h poststress corticosterone concentrations and cell proliferation. This is most likely due to the fact that plasma corticosterone was collected 24 h after forced swimming, which is too late to establish a direct correlation between these parameters. Nevertheless, inhibitory effects of corticosterone itself on adult proliferation are well established (20, 24, 60–63). In the rat, blockade of glucocorticoids synthesis by adrenalectomy increases both dentate gyrus proliferation and apoptosis (20, 64, 65), whereas exogenous corticosterone application again reduces these parameters (24, 62). Treatment with a GR or a MR agonist also reduced dentate gyrus proliferation in adrenalectomised rats (37). Despite these findings, the mechanism through which stress or corticosterone affects new cell birth remains poorly understood.

The population of adult-generated precursor cells generally lack GR expression for the first few days (66, 67). Therefore, direct effects of corticosterone via GRs on precursor cells are rather unlikely. Because the granular cell layer contains high concentrations of GR, corticosteroid signalling has been proposed to occur indirectly via, for example, neighbouring mature granule cells or glia cells. Alternatively, growth factors, specific cell cycle regulatory proteins (68), vascular elements, or upstream mechanisms involving the NMDA receptor-mediated excitatory pathway have been implicated as well (69–72). For example, blockade of NMDA receptors enhances dentate gyrus cell proliferation (73), whereas activation of NMDA receptors inhibit cell proliferation in rodents (69). However, in the present study, no line-difference was found for hippocampal NMDA receptor binding, whereas quantitative analysis of GR-immunoreactivity in naïve LAL and SAL mice revealed significantly higher GR levels in the dentate gyrus, but not in the CA1 area, of LAL mice. Taken together, the enhanced corticosterone response and the higher GR expression in the dentate gyrus suggest a role for corticosterone in the selective reduction in hippocampal cell proliferation after forced swimming in LAL mice. Clearly, more studies are needed to demonstrate the causal role of corticosterone in regulating the stress-induced hippocampal cell proliferation rate in these mouse lines.

Interestingly, SAL mice failed to show a swim stress-induced decrease in cell proliferation. Elsewhere, dentate gyrus cell proliferation appeared also resistant to acute stress exposure (63, 74). In addition to a possible role for a line-difference in corticosterone response and GR levels, this stress-resistant effect on cell proliferation in SAL mice might have been mediated by simultaneous activation of positive regulators of adult hippocampal cell proliferation. For example, activation of 5-HT_{1A} receptors is associated with an up-regulation of adult hippocampal cell proliferation (75). Adult hippocampal cell proliferation was increased after administration of fenfluramine, which causes the release of 5-HT, and after 8-OH-DPAT stimulating 5-HT_{1A} receptors (76). In addition, three 5-HT_{1A} receptor antagonists (WAY-100635, NAN-190, p-MPPI) were demonstrated to reduce basal cell proliferation rate (77). In this respect, it is of interest to note that SAL mice have higher hippocampal 5-HT_{1A} receptor expression and binding capacity (33, 78), higher

hippocampal 5-HT responsiveness (79) and higher brain 5-HT turnover (33) than LAL mice. Although further research is required, a potential role of the hippocampal 5-HT_{1A} receptor in preventing the stress-induced decrease in cell proliferation in SAL mice is assumed.

It should be noted that, because the SAL males are highly aggressive, it is not possible to house two males together. To prevent social isolation, SAL and LAL mice are commonly housed in pairs with a female. Given the established robust and persistent behavioural and neuroendocrine differences between SAL and LAL mice housed in this way for several generations (9, 12), it is unlikely that these housing conditions have significantly affected our present findings.

The SAL and LAL mice represent the extremes in interindividual differences in coping style and stress responsiveness. This amplification of interindividual differences provides useful information that may help to understand why some individuals are more likely to suffer from stress-related disorders than others under seemingly similar conditions. Previous studies have reported differences in adult hippocampal cell proliferation rate between particular strains of mice (80–83). We extend this finding by showing differences in baseline and stress-induced hippocampal cell proliferation in two lines of wild house mice. A recent study in mice reported that individual differences in hippocampal cell proliferation are related to the level of defensive behaviour displayed during social defeat (84). Taken together, these and our results suggest that interindividual differences in proliferation are associated with individual differences in certain behavioural phenotypes. In support, a correlation has been found between genetically determined baseline adult neurogenesis and the ability to acquire spatial memory on the water maze task or vice versa (85, 86).

In conclusion, the present data indicate that stressful stimuli selectively suppressed adult cell proliferation in the dentate gyrus of LAL mice, probably in part through an action mediated by corticosterone, whereas SAL mice were found to be resistant. This differential susceptibility to stress-induced changes in adult cell proliferation suggests that hippocampal structural plasticity may be involved in the established line-differences in coping style and behavioural adaptation during stress exposure.

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